APPENDIX C

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Adenocarcinoma Cells Are Targeted by the New GnRH-PE$_{66}$ Chimeric Toxin through Specific Gonadotropin-releasing Hormone Binding Sites*

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Luteinizing hormone-releasing hormone, also termed gonadotropin-releasing hormone (GnRH), accounts for the hypothalamic-pituitary gonadal control of human reproduction. The involvement of GnRH has been demonstrated in several carcinomas of hormone-responsive tissues. Exploiting this common feature, we constructed a *Pseudomonas* exotoxin (PE)-based chimeric toxin (GnRH-PE$_{66}$) aimed at targeting those cancer cells bearing GnRH binding sites. We report here the strong growth inhibition and killing of a surprisingly wide variety of cancers, confined to the adenocarcinoma type. These cancer cells arising from hormone-responsive tissues, as well as non-responsive ones, express specific GnRH binding sites as indicated by the marked killing of ovarian, breast, endometrial, cervical, colon, lung, hepatic, and renal adenocarcinoma. This cytotoxicity is specific as it could be blocked upon addition of excess GnRH. The specificity of GnRH-PE$_{66}$ chimeric toxin was also confirmed by GnRH binding assays, and its ability to prevent the formation of colon cancer xenografts in nude mice is presented. Although the functional role of specific GnRH binding sites in human carcinomas remains obscure, GnRH-PE$_{66}$ displays considerable targeting potential and its use as a therapeutic agent for cancer should be considered.

Gonadotropin-releasing hormone (GnRH)[1] is a decapeptide that is normally synthesized by the hypothalamic neurons and secreted into the hypophysioportal circulation via portal vessels. It is synthesized as a larger peptide and matures through proteolytic processing and amidation at its C-terminal glycine. Upon reaching the anterior pituitary gland, GnRH selectively stimulates the gonadotroph cells to release luteinizing hormone (LH) and follicle-stimulating hormone, thus playing a central role in the neuroendocrine control of human reproduction.

The involvement of GnRH has been demonstrated in several carcinomas (1). GnRH-specific binding sites have been reported in some solid tumors, as well as in established cell lines (2–6), although the functional role of these binding sites in human neoplasms remains obscure.

Numerous analogs have been developed mainly to overcome the very short half-life of GnRH (7, 8) and to enhance its affinity for the GnRH receptor (9). These GnRH analogs suppress the growth of various tumor cells *in vitro* and *in vivo*. The use of GnRH analogs has been proposed for treatment of various endocrine-dependent cancers, like breast (2), prostatic (10), pancreatic (11), endometrial (3, 12), and ovarian (13, 14) carcinoma. Nonetheless today, only prostatic cancer is frequently challenged with such treatment; however, relapses occur in most patients, after their malignancy becomes androgen-insensitive (15).

The use of toxin-carrying molecules for targeting specific cells of interest has been successfully implemented (16, 17). One of the most widely applied, *Pseudomonas* exotoxin A (PE), acts by irreversibly arresting protein synthesis in eukaryotic cells. The toxin inactivates elongation factor 2 through ADP-ribosylation (18), causing cell death. A mutated form of PE, lacking its binding ability (PE$_{66}$4Glu) (19) and fused to an antigen that internalizes upon binding through a specific cell surface receptor, as in the case of GnRH, could be a powerful tool in the fight against cancer.

In this study, construction of the GnRH-PE$_{66}$ chimeric toxin, its overexpression, and its purification are described. The cytotoxic effect of GnRH-PE$_{66}$ on cell lines and primary cultures of malignant specimens as well as its ability to prevent the growth of colon carcinoma xenografts in nude mice are presented.

Recent data, demonstrating a response to GnRH analogs by nongynecological tumors, *e.g.* cancer of the pancreas and kidney, as well as hepatoma (6, 11, 20), led us to examine the possibility that this may be a more widespread phenomenon among neoplasmic diseases. We show here that most adenocarcinoma cells, including those of the lung, colon, breast, ovary, kidney, liver, endometrium, and cervix, are all selectively killed by the GnRH-PE$_{66}$ chimeric toxin.

EXPERIMENTAL PROCEDURES

[α-^{35}S]dATP, [^{3}H]leucine, [^{14}C]NAD, and (3-[^{125}I]iodotyrosyl^{5})LHRH were purchased from Amersham (UK).

Escherichia coli strain DH5α (Stratagene) was used for all plasmid transformations and propagations. Strain BL21(λDE3), which carries a T7 RNA polymerase gene in lysogenic and inducible form, was used for expression of the chimeric proteins (21).

Restriction and modifying enzymes were obtained from Boehringer Mannheim (Germany). DNA sequencing was performed with a Sequenase kit (U. S. Biochemical Corp.). All media and antibiotics were obtained from Biological Industries (Beit Haemek, Israel).

Plasmid Construction—A plasmid vector carrying the mutated full-length PE gene (pJY3A1136–1,3) (19) was cut with *Nde*I and *Hin*dIII. The insert was a 36-base pair synthetic oligomer consisting of the GnRH coding sequence, with tryptophan replacing glycine as the sixth amino acid, flanked by *Nde*I (5′ end) and *Hin*dIII (3′ end) restriction sites (Fig. 1). The resulting TGnRH-PE$_{66}$ plasmid was confirmed by restriction endonuclease digestion and DNA sequence analysis.

Protein Expression and Purification—*E. coli* strain BL21(λDE3) carrying plasmid TGnRH-PE$_{66}$, pJY3A1136–1,3, or PIS2 (an unrelated

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[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; PE, *Pseudomonas* exotoxin; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

80-base pair sequence fused to PE$_{66}$4Glu), was grown in LB medium containing ampicillin (100 μg/ml).

After reaching an A_{600} value of 1.5–1.7, the cultures were induced for 90 min at 37 °C with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were collected by centrifugation, and the pellet was stored at −70 °C for several hours. The frozen pellet was thawed and suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.2 mg/ml lysozyme), followed by sonication (3 × 30 s) and centrifugation at 35,000 × *g* for 30 min. The supernatant (soluble fraction) was removed, and the pellet (insoluble fraction) was suspended and stirred on ice in denaturation buffer (6 M guanidinium-HCl, 0.1 M Tris-HCl, pH 8.6, 1 mM EDTA, 0.05 M NaCl, and 10 mM dithiothreitol). After an additional centrifugation, the protein solution was diluted 1:100 in refolding buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M NaCl, 0.25 M L-arginine, and 5 mM dithiothreitol), and kept at 4 °C for 48 h. The refolded protein solution was either dialyzed against phosphate-buffered saline (PBS) and tested directly in cytotoxic assays, or further purified by ion-exchange and gel filtration, as follows. The refolded protein solution was diluted with TE$_{20}$ buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a conductivity of 8 millisiemens/cm. Following addition of DEAE-Sepharose Fast Flow (Pharmacia, Sweden) and stirring for 30 min at 4 °C, the solution was packed into a 2.5 × 15-cm glass column and washed with 80 mM NaCl in TE$_{20}$ until an A_{280} value of 0 was obtained. The chimeric toxin was eluted by a linear gradient of 2 × 200 ml of 0.08–0.35 M NaCl in TE$_{20}$. The peak fractions were concentrated, using a Stirred Cell (Sigma) in the presence of 0.5 M L-arginine, and passed through a 2.5 × 120-cm Sephacryl S-200 HR (Pharmacia, Sweden) column in 0.15 M potassium phosphate buffer, pH 6.0, containing 0.5 M NaCl. The peak fractions were collected and dialyzed against PBS at 4 °C, and kept in aliquots at −20 °C.

Samples of the various protein fractions were Western-blotted and analyzed with αPE, using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA), according to the manufacturer's instructions.

ADP-ribosylation Assay—ADP-ribosylation activity was measured by incubating the various protein preparations with [^{14}C]NAD and wheat germ extracts enriched in elongation factor 2, as described in Ref. 22.

Cell Lines—Breast carcinoma MDA MB-231 and colon carcinomas SW-48 and HT-29 were kindly provided by Aviva Horwitz (Hadassah Hospital, Jerusalem, Israel). Ovarian carcinoma OVCAR3 was kindly provided by Ira Pastan (National Institutes of Health, Bethesda, MD). Colon carcinoma Caco 2, hepatocarcinoma HepG2, cervical adenocarcinoma HeLa, and transformed primary embryonal kidney 293 were kindly provided by Ruth Shemer (Hebrew University, Jerusalem, Israel). Bladder carcinoma J-82 and T-24A, rhabdomyosarcoma A-204, and breast carcinoma MCF-7 were kindly provided by Abraham Hochberg (Hebrew University, Jerusalem, Israel).

Unless specified, all cell lines were maintained in RPMI 1640 medium, cultured in 100-mm Petri dishes, and grown in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

HepG2 and Caco2 were maintained in Eagle's minimal essential medium, and HeLa and 293 were maintained in Dulbecco's modified Eagle's medium. T-24A, J-82, NT-2, and A-204 were maintained in Dulbecco's modified Eagle's medium, Ham's F-12 (1:1). All media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Primary Cultures—Fresh tissue specimens were taken from cancer patients undergoing therapeutic debulking procedures. Control specimens were obtained from donors and patients undergoing diagnostic or therapeutic procedures for nonmalignant diseases.

All tissue specimens were washed several times with Leibovitz (L15) medium, minced, and subjected to enzymatic proteolysis for 2 h at 37 °C

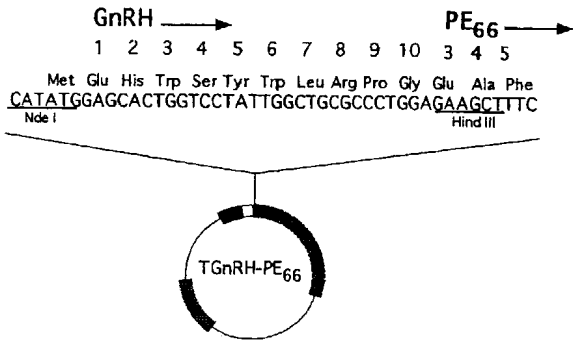

FIG. 1. **Construction of the TGnRH-PE$_{66}$ plasmid that encodes the GnRH-PE$_{66}$ chimeric toxin.** Ten amino acids of a GnRH analog were inserted in the *NdeI-Hind*III sites of the mutated full-length *Pseudomonas* exotoxin gene. *Black area*, T7 promoter; *white area*, GnRH analog peptide; *dark gray area*, ampicillin^R; *light gray area*, PE$_{66}$4glu. The *numbers* represent the corresponding amino acids.

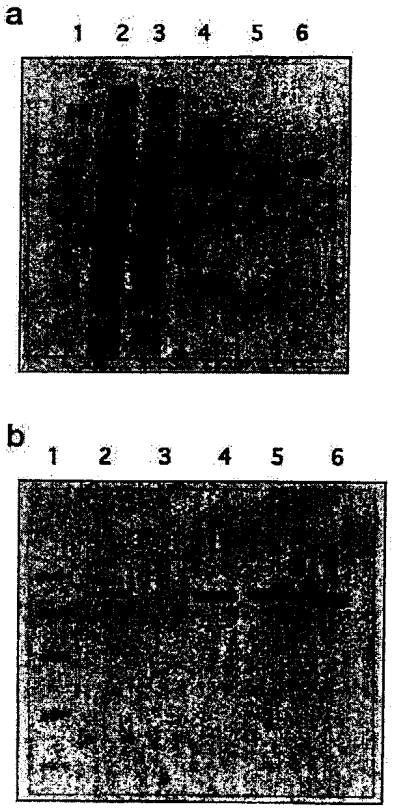

FIG. 2. **SDS-PAGE analysis of GnRH-PE$_{66}$ purification.** GnRH-PE$_{66}$ was overexpressed in *E. coli* BL21(λDE3), purified by separation of the insoluble fraction by ion exchange column and gel filtration. *Panel a*, SDS-PAGE gel stained with Coomassie Blue. *Panel b*, immunoblot using αPE. *Lane 1*, standard molecular weight marker; *lane 2*, whole cell extract; *lane 3*, soluble fraction; *lane 4*, insoluble fraction; *lane 5*, after DEAE-Sepharose column; *lane 6*, after Sephacryl-S-200 HR column.

TABLE I
Purification of GnRH-PE$_{66}$

Purification of GnRH-PE$_{66}$ was assessed by ADP-ribosylation activity, indicating the enzymatic capability of the chimeric toxin. All measurements were done in triplicate.

Fraction	Protein concentration	Total protein	Specific activity	Total activity	Yield		Degree of purification
					Activity	Protein	
	mg/ml	*mg*	*cpm/mg*	*10^6 cpm*	%	%	
Whole cell extract	2.685	160	9.6	1537	100	100	1
Insoluble	0.273	83.5	120	10037	653	52	12.5
After DEAE-Sepharose	0.158	7.1	276.3	1962	127.6	4.4	28.8
After Sephacryl S-200 HR	0.063	2.2	358.4	788	51	1.4	37.3

with gentle shaking in Leibovitz medium containing collagenase type I (200 units/ml), hyaluronidase (100 units/ml) (Sigma), penicillin (1000 units/ml), streptomycin (1 mg/ml), and amphotericin B (2.5 μg/ml).

Tissue preparations were centrifuged for 10 min at 200 × *g*, and the pellets were suspended in RPMI 1640 medium containing all supplements and plated in 100-mm Petri dishes. After 1–3 weeks, when the cultures had reached a density of ~8 × 10^6 cells/plate, histopathological diagnoses and cytotoxic assays were performed.

Leukocyte Cultures—Normal leukocytes from peripheral blood and bone marrow aspirates from control healthy donors were obtained and processed for cytotoxic assays as described in Ref. 23.

Histopathological Diagnosis—To determine the origin of the primary cultures, cells were stained as follows: 10,000 cells were plated with the aid of a Cytospin (Shandon Inc.), on a microscope slide, and incubated for several min at room temperature. Dried slides were fixed by soaking in −20 °C cold methanol for 15 min and in −20 °C cold acetone for several seconds. Slides were kept at −20 °C until stained. Immunoperoxidase staining was performed with a Histostain kit (Zymed Laboratories Inc.), according to the manufacturer's instructions, using anti-desmin and anti-keratin antibodies to distinguish fibroblasts from epithelial cells. To confirm the authentic malignant origin of the cultures, a fresh cytologic sample and a formalin-fixed histologic sample from each patient were stained with a specific anti-tumor marker antigen. Anti-CA 19-9 was used to identify colon carcinoma, anti-CA 15-3 for breast carcinoma, and anti-CA 125 for ovarian carcinoma (Cis-Bio Int.).

Binding Assays—Specific binding and displacement of GnRH was studied in plasma membrane fractions of SW-48 cells as described in Ref. 24. The cells were homogenized in ice-cold assay buffer (10 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 0.15% bovine serum albumin, 1 mM EDTA) and centrifuged at 250 × *g* for 15 min at 4 °C. The pellet was discarded, the supernatant was centrifuged at 20,000 × *g* for 30 min at 4 °C, and the plasma membrane pellet was resuspended in cold assay buffer.

Aliquots containing 70 μg of plasma membrane protein (determined according to Bradford) (25) in a final volume of 100 μl, were incubated for 2 h on ice with 6 × 10^{-6} M (240,000 cpm) ^{125}I-GnRH in the presence or absence of (10^{-4} to 10^{-10} M) unlabeled authentic GnRH peptide and analog (des-Gly^{10}-[D-Ala^6]LHRH) or (2.5 × 10^{-5} to 10^{-9} M) GnRH-PE$_{66}$ chimeric toxin. Following incubation, samples were washed through Whatman GF/C filters with 10 ml of cold assay buffer and counted in a γ counter.

All binding studies were performed in triplicate. Nonspecific binding was determined in the presence of 10^{-4} M unlabeled GnRH or analog.

Cytotoxicity Assays—The cytotoxic activity of GnRH-PE$_{66}$ was tested on various primary cultures and cell lines. Cells (10^4 in 0.2 ml culture medium) were seeded in 96-well microplates, and 24 h later various concentrations of GnRH-PE$_{66}$ were added. Mutated PE$_{66}$ and PIS2 protein (both obtained as described above for GnRH-PE$_{66}$) served as controls. After a 24-h incubation (day 2), [^{3}H]leucine (5 μCi (37 kBq)/well) was added overnight.

The plates were then stored at −70 °C for several hours, followed by quick thawing at 37 °C. The cells were harvested on filters, and the incorporated radioactivity was measured with a β counter. The results are expressed as the percent incorporation of the control experiments in which the cells were not exposed to protein.

In Vivo GnRH-PE$_{66}$ Toxicity—Toxicity was assessed in two different assays. In the first, groups of three C57BL/J6 × BALB/c female mice were intraperitoneally administered with a single dose of 5, 10, or 25 μg/mouse GnRH-PE$_{66}$ and the animals were observed for 5 days. In the second assay, groups of three 6–8-week-old *nude/nude* female mice were treated intraperitoneally twice daily with 5, 10, or 12.5 μg/mouse/day of GnRH-PE$_{66}$ for 10 days. All injections were performed in a volume of 0.5 ml, and toxicity studies were repeated twice.

Establishment of a Tumor Model and Antitumor Experiments in Vivo—Adult 6–8-week-old nude/nude mice were injected subcutaneously with 2.2 × 10^6 Caco2 colon carcinoma cells in 100 μl of PBS. Treatment started 36 h later. Groups of 10 mice were treated with one of the following: 5 and 10 μg/day/mouse of purified GnRH-PE$_{66}$, 0.176 μg/day/mouse GnRH hormone and an equal volume of PBS, injected intraperitoneally every 12 h for 10 days in a volume of 0.5 ml. Three days after termination of treatment, mice bearing tumors were killed, and tumors and various organs were pathologically examined. Mice that did not develop tumors were observed for an additional month.

RESULTS

Construction, Expression, and Purification of GnRH-PE$_{66}$ Chimeric Toxin—An oligonucleotide encoding the 10 amino acids of the GnRH analog (tryptophan replacing glycine as the sixth amino acid) was ligated upstream to a mutated form of *Pseudomonas* exotoxin, PE$_{66}$4Glu (19), to generate GnRH-PE$_{66}$ (Fig. 1). Following transformation of *E. coli* BL21(λDE3) cells with the TGnRH-PE$_{66}$ plasmid, expression of the fusion gene was controlled by the bacteriophage T7 late promoter (21).

Chimeric proteins tend to accumulate within the insoluble

TABLE II

The effect of GnRH-PE$_{66}$ on various cell lines

Partially purified GnRH-PE$_{66}$ was tested for inhibition of protein synthesis on various carcinoma cell lines. The numbers represent the amount of GnRH-PE$_{66}$ causing 50% cell death.

Cell lines	Origin	ID$_{50}$
		μg total protein/well
Caco2	Colon carcinoma	0.4
HT-29	Colon carcinoma	1.2
SW-48	Colon carcinoma	0.3
OVCAR3	Ovarian carcinoma	3
MCF-7	Breast carcinoma	0.7
MDA MB-231	Breast carcinoma	2.3
HeLa	Cervix adenocarcinoma	1.8
293	Renal cell carcinoma	0.3
HepG2	Hepatocarcinoma	0.3
J-82	Bladder carcinoma	No effect
T-24A	Bladder carcinoma	No effect
A-204	Rhabdomyosarcoma	No effect

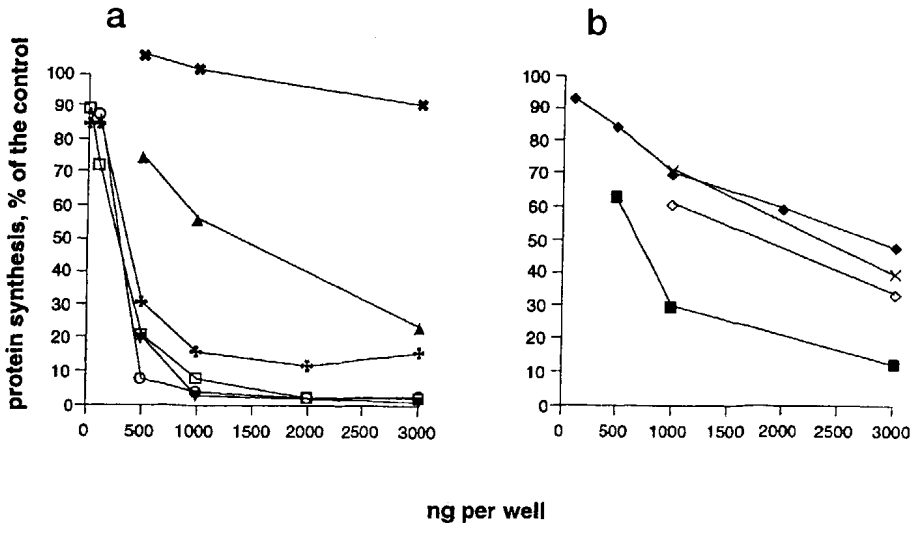

FIG. 3. **The effect of the partially purified GnRH-PE$_{66}$ on various cell lines.** *Panel a*, non-hormone-responsive tumors; *panel b*, hormone-responsive tumors. ✱, J-82 bladder carcinoma; +, □, and ▲, Caco2, SW-48, and HT-29 colon carcinoma; ○, HepG2 heptocarcinoma; ▼, 293 transformed embryonal kidney; × and ■, MDA-MB-231 and MCF-7 breast carcinoma; ♦, OVCAR3 ovarian carcinoma; ◇, HeLa cervical adenocarcinoma.

cellular fraction in the form of inclusion bodies, thereby facilitating partial purification of the recombinant protein by denaturation and renaturation of the insoluble fraction. The partially purified fraction (Fig. 2*a*, *lane 3*) was highly enriched with the GnRH-PE$_{66}$ chimeric toxin, enabling us to examine its cytotoxic ability *in vitro*. Further purification by ion-exchange and gel filtration yielded a protein preparation of >97% purity.

Analysis of the purification steps by SDS-PAGE revealed a major band with an expected molecular mass of 67 kDa, corresponding to the chimeric protein (Fig. 2). Immunoblotting with αPE confirmed these data (Fig. 2*b*). Measurement of ADP-ribosylation activity verified the enzymatic capability of GnRH-PE$_{66}$ and the high degree of purification obtained (Table I).

Effect of the GnRH-PE$_{66}$ Chimeric Protein on Cell Lines—The cytotoxic activity of the partially purified GnRH-PE$_{66}$ on different malignant cell lines was assessed by measuring the inhibition of protein synthesis. The chimeric protein was found to kill cells in a dose-dependent manner, with considerable variation between cell lines. Carcinoma cell lines of hormone-responsive origin ovary, breast, and cervix exhibited ID_{50} values between 0.7 and 3 μg/well (Table II and Fig. 3*b*). Surprisingly, the toxin had a greater effect on the non-hormone-responsive lines, *i.e.* colon, renal, and hepatic carcinoma, with ID_{50} values ranging between 0.3 and 1.2 μg/well (Table II and Fig. 3*a*). Although cytotoxicity was measured by inhibition of amino acid incorporation, cell death was reflected in cell number and/or cell necrosis 24 h following addition of the chimeric protein. Cell lines established from other cancers, such as A-204 rhabdomyosarcoma and T-24A and J-82 bladder carcinoma, did not respond to the chimeric toxin treatment (Table II).

To confirm the specificity of GnRH-PE$_{66}$ activity, two other PE-based recombinant proteins, expressed and extracted under the same conditions, were used as controls. No substantial growth inhibition was exerted by either PE$_{66}$4Glu or PIS2 proteins (data not shown).

Upon testing the ability of the highly purified GnRH-PE$_{66}$ chimeric toxin to target various cancer cell lines, a 3–4-fold increase was obtained (data not shown). The toxin had no effect on the two bladder carcinoma cell lines, similar to the results obtained with the partially purified chimeric toxin preparations (Table II).

The cytotoxicity of highly purified GnRH-PE$_{66}$ was also assayed in the presence of excess GnRH hormone. GnRH was found to inhibit strongly the cytotoxic effect of the fusion toxin (Fig. 4). Addition of other nonrelevant peptides, similar in size and content of hydrophobic amino acids, had no effect on GnRH-PE$_{66}$-mediated cytotoxicity (data not shown).

Effect of the GnRH-PE$_{66}$ Chimeric Toxin on Primary Cultures—To study the cytotoxicity of our chimeric toxin against cells resembling the original *in vivo* tumors as closely as possible, primary cultures were established from biopsies of various cancer patients, as well as control specimens obtained from healthy donors and patients with benign diseases. The malignant cells presented a clear picture of dose-dependent growth inhibition in response to GnRH-PE$_{66}$ (partially purified fraction) treatment (Fig. 5, *a–f*) often with visible cell necrosis. Ovarian, breast, and endometrial carcinoma displayed ID_{50} values ranging between 0.85–3, 1–3, and 1 μg/well, respectively. Non-hormone-responsive cells of colon, kidney, and lung carcinoma displayed ID_{50} values ranging between 0.9–3, 1.3, and 1.6 μg/well, respectively (Table III). In cases where biopsies of metastases were available, primary cultures were established. The cytotoxic effect on these cultures displayed a pattern similar to that in the corresponding primary tumor cultures (data not shown). All these tumors were independ-

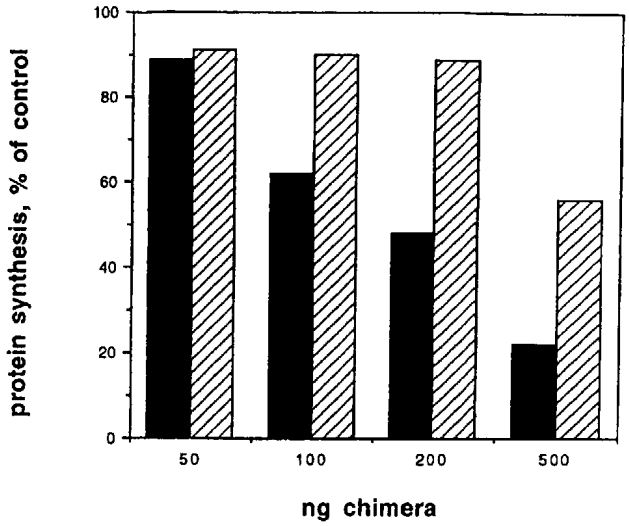

FIG. 4. **Inhibition of GnRH-PE$_{66}$ cytotoxicity by GnRH.** CaCo2 colon carcinoma cells were incubated with 35 μg(1.5×10^{-4} M) of GnRH, in a standard cytotoxic assay. *Solid bars*, purified GnRH-PE$_{66}$ alone; *hatched bars*, in the presence of the GnRH hormone.

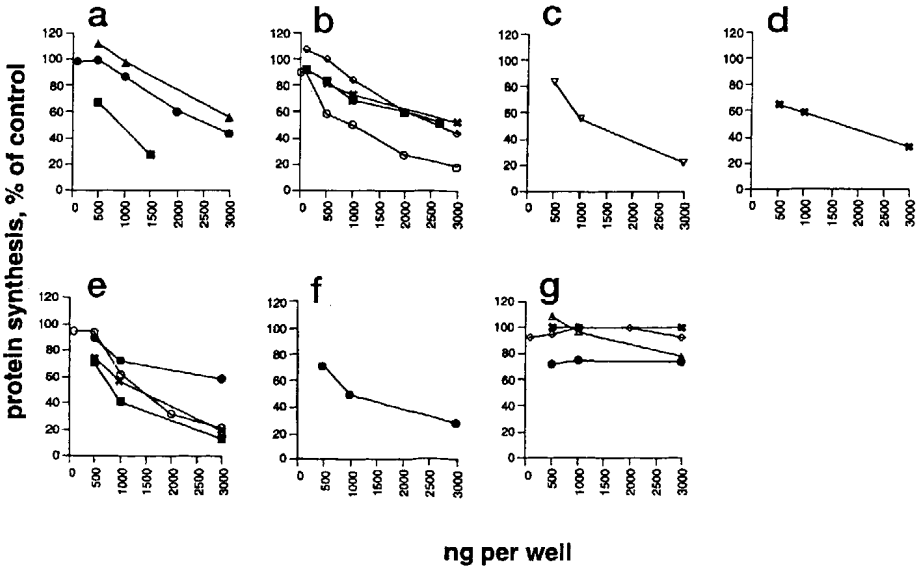

FIG. 5. **The effect of partially purified GnRH-PE$_{66}$ on various primary cultures.** Each line represents a primary culture established from a different patient. *Panel a*, colon carcinoma; *panel b*, breast carcinoma; *panel c*, renal cell carcinoma; *panel d*, lung adenocarcinoma; *panel e*, ovarian carcinoma; *panel f*, endometrial carcinoma; *panel g*, normal primary cultures. ●, skin fibroblasts; ✖, leukocytes; △, bone marrow; ◇, colon.

TABLE III

The effect of GnRH-PE$_{66}$ on various primary cultures

Partially purified GnRH-PE$_{66}$ was tested for inhibition of protein synthesis on various primary cultures. The cultures were established from primary tumors or/and metastasis biopsies. The numbers represent the amount of GnRH-PE$_{66}$ causing 50% cell death.

Origin	Primary tumor ID_{50}	Metastasis ID_{50}
	μg total protein/well	
Colon		
adenocarcinoma	0.9	
adenocarcinoma	—[a,b]	
adenocarcinoma	3	
adenocarcinoma	2.1	2
adenocarcinoma		2
Renal		
Clear cell carcinoma (adeno)	1.3	
Endometrium		
adenocarcinoma	1	
adenocarcinoma	—[a,b]	
Ovary		
adenocarcinoma	0.85	
adenocarcinoma	—[a,b]	
adenocarcinoma	1.4	0.8
adenocarcinoma	>3	3
adenocarcinoma	1.4	1.4
Breast		
adenocarcinoma	3	
adenocarcinoma	1	1.4
adenocarcinoma	2.6	
adenocarcinoma	3	
Lung		
adenocarcinoma	1.6	
Other tumors		
Lung squamous carcinoma	—	
Lung squamous carcinoma	—	
Transitional cell carcinoma (bladder)	—	
Transitional cell carcinoma (bladder)	—	
Granulosa cell tumor	—	
Non-Hodgkin's lymphoma	—	

[a] —, increasing amounts of GnRH-PE$_{66}$ did not cause any significant inhibition of protein synthesis.

[b] Primary cultures established from recurrent tumors.

ently classified as adenocarcinomas by a pathologist.

Non-adenocarcinoma primary cultures originated from two bladder carcinomas, two squamous lung carcinomas, a granulosa cell tumor, and a Non-Hodgkin's lymphoma, did not respond to the GnRH-PE$_{66}$ treatment (Table III). Normal and malignant biopsies taken from the same patients enabled us to furthur prove GnRH-PE$_{66}$ specificity. Treatment of the right healthy ovary with our chimeric toxin showed no effect, while the left malignant one clearly displayed dose-dependent growth inhibition (Fig. 6*b*). A similar response was evident when benign and malignant biopsies from the same colon were tested (Fig. 6*a*). GnRH-PE$_{66}$ was also tested on cultures of benign colon, peripheral blood, bone marrow, and skin fibroblasts from healthy donors. The addition of increasing amounts of the partially purified chimeric protein did not result in any measurable dose-dependent killing (Fig. 5*g*).

In working with epithelial primary cultures, the genuine epithelial origin of the cells must be verified. The tendency of primary cultures to lose their epithelial morphology has been described in carcinomas of the ovarian epithelium (26) and in bladder transitional-cell carcinoma (27). To ascertain the absence of any substantial "contaminating" fibroblasts, differential staining was performed. Anti-desmin (for fibroblast) *versus* anti-keratin (for epithelium) staining of the primary cultures (data not shown) indicated that the vast majority (80–100%) of the cells were indeed epithelial, even in cases where the cultures exhibited a fibroblast-like shape.

Further confirmation was achieved by staining with specific anti-tumor marker antigens. Formalin-fixed sections from the

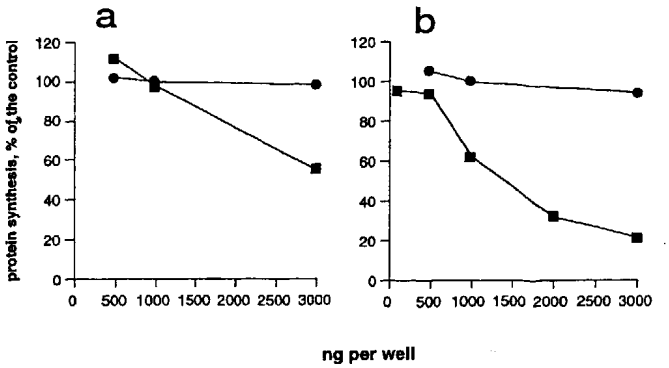

FIG. 6. **The effect of partially purified GnRH-PE$_{66}$ on benign and malignant primary cultures established from the same patient.** *Panel a*, colon; *panel b*, left and right ovary. ●, benign; ■, malignant.

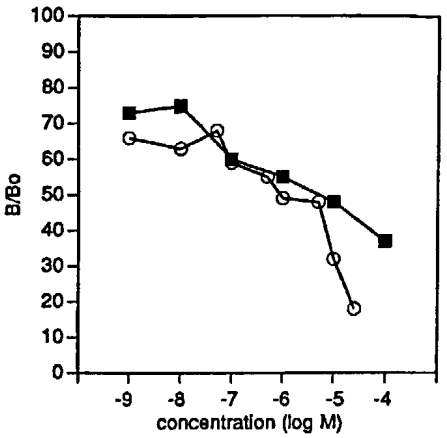

FIG. 7. **Displacement of [^{125}I]GnRH bound to membranes of SW-48 cells by: ○ partially purified GnRH-PE$_{66}$. ■**, GnRH analog (des-Gly^{10}-[D-Ala^{6}]LHRH). *B*, amount of [^{125}I]GnRH (in cpm) bound to the membrane fraction in the presence of the competitor (GnRH-PE$_{66}$, GnRH, GnRH analog) at the indicated concentration. *Bo*, amount of [^{125}I]GnRH (in cpm) bound to the membrane fraction in the absence of any competitor.

original tumors and the tested primary culture cells displayed the same pattern and intensity of staining (results not shown).

GnRH-PE$_{66}$ Binding Assay—Although GnRH binding sites have been demonstrated in a number of solid tumors and in various carcinoma cell lines, their existence in colon or lung carcinoma has not been documented. The addition of increasing concentrations of partially purified GnRH-PE$_{66}$ chimeric toxin resulted in dose-related displacement of the ^{125}I-GnRH bound to plasma membrane fraction of SW-48 colon carcinoma cells. The unlabeled analog des-Gly^{10}-[D-Ala^{6}]LHRH produced similar results (Fig. 7), as did the original GnRH peptide (results not shown).

As can be seen in Fig. 7, binding of labeled GnRH to the cells was specific and displacement by GnRH-PE$_{66}$ was as efficient as that by the GnRH analog peptide. There was 37% nonspecific binding. Similar results of specific binding were obtained with the HT-29 colon carcinoma cells (results not shown).

Toxicity of GnRH-PE$_{66}$ in Mice—Groups of three C57BL/J6 × BALB/c female mice were injected intraperitoneally with single doses of purified GnRH-PE$_{66}$ and observed for mortality. One animal died following injection of 10 μg, and two died after 25 μg of the chimeric toxin had been administered (Table IV). The LD_{50} of GnRH-PE$_{66}$ was estimated as ~15 μg/mouse.

In evaluating toxicity in the full -assay format, groups of three *nude/nude* female mice were injected intraperitoneally twice daily with purified GnRH-PE$_{66}$ for 10 days. Since no

TABLE IV
Toxicity of GnRH-PE$_{66}$ in C57BL/J6 × BALB/c mice

Mice were injected intraperitoneally with a single dose of the indicated amounts of purified GnRH-PE$_{66}$, and the number of dead animals was determined 5 days later.

Dose	GnRH-PE$_{66}$
μg/mouse/day	*no. of dead/no. of injected*
5	0/3
10	1/3
25	2/3

TABLE V
Toxicity of GnRH-PE$_{66}$ in nude/nude mice

Mice were injected intraperitoneally twice a day for 10 days with the indicated amounts of purified GnRH-PE$_{66}$. The number of dead mice was determined 3 days after termination of treatment.

Dose	GnRH-PE$_{66}$
μg/mouse/day	*no. of dead/no. of injected*
5	0/3
10	0/3
12.5	1/3

deaths were recorded at a dosage of 10 μg or less (Table V), we chose this dose as the maximal amount to be given *in vivo*.

Effect of GnRH-PE$_{66}$ Treatment on Tumor Xenografts in Nude Mice—In an attempt to determine the ability of purified GnRH-PE$_{66}$ chimeric toxin to target cancer cells *in vivo*, we treated nude mice, induced to develop subcutaneously solid tumors, with our new chimeric toxin. These mice develop solid tumors between 4 and 5 days following subcutaneous injection of 2.2×10^6 Caco2 colon carcinoma cells. Treatment of the mice 36 h after cell injection, prevented tumor development in a dose-dependent manner (Fig. 8). A dosage of 5 μg/day/mouse GnRH-PE$_{66}$ inhibited tumor growth in 40% of the mice, while 10 μg/day/mouse prevented appearance of the tumors in 80% of the animals. In the control groups, which received only PBS or the GnRH hormone, 80% of the mice developed tumors (Fig. 8).

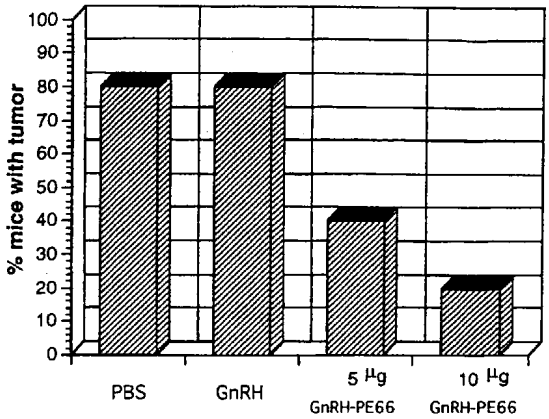

FIG. 8. **The effect of purified GnRH-PE$_{66}$ on xenograft formation in nude mice.** Caco2 colon carcinoma cells (2.2×10^6) were injected subcutaneously into nude mice. After 36 h groups of mice (n = 10) were injected intraperitoneally every 12 h for 10 days with the following doses: 5 and 10 μg/day/mouse of purified GnRH-PE$_{66}$, an equivalent molar amount (0.176 μg/day/mouse) of the GnRH hormone, and an equal volume of PBS. On day 13, mice bearing tumors were sacrificed and the tumors were collected and examined.

DISCUSSION

The major findings of this study are the surprisingly widespread presence of GnRH binding sites in human neoplasms (in most of the adenocarcinomas we tested) and the impressive ability of our new GnRH-PE$_{66}$ chimeric toxin to target and kill such cancer cells.

Consistent with the results of previous studies demonstrating GnRH binding sites in hepatic HepG2 (6), ovarian OVCAR3 (14), and breast MDA MB (28) carcinoma cell lines, we found that these cultures were markedly affected by the chimeric toxin (Fig. 3, *a* and *b*). However, the most unexpected observation was the significant growth inhibition and cell killing of non-hormone-responsive tumors, namely colon, kidney, and lung adenocarcinoma. This phenomenon, confined to adenocarcinoma type of cancers, was observed in various cell lines as well as in different primary cultures treated with the new GnRH-PE$_{66}$ chimeric toxin (Figs. 3 and 5).

Of the 18 adenocarcinomas tested, 15 responded to the chimeric toxin treatment (Table III). The three primary cultures that did not respond to GnRH-PE$_{66}$ treatment were all established from recurrent tumors, in contrast to the primary tumors used for the other 15. All cell lines and primary cultures that originated from tumors other than adenocarcinomas, whether or not of epithelial origin, were unaffected by GnRH-PE$_{66}$ treatment.

The specificity of our chimeric toxin is manifested by the resistance of normal cells as well as nonadenocarcinoma cells to GnRH-PE$_{66}$ treatment (Figs. 5*g* and 6) and its remarkable efficiency in prevention of colon carcinoma xenograft formation in nude mice (Fig. 8). Addition of GnRH peptide strongly interfered with the GnRH-PE$_{66}$ effect (Fig. 4). Moreover, the ability of plasma membrane fractions from SW-48 colon carcinoma cells to bind GnRH and its efficient displacement by GnRH-PE$_{66}$ (Fig. 7) not only demonstrate the existence of GnRH binding sites on these cells, but also prove their ability to bind our new chimeric toxin. Bearing in mind the large differences in molecular mass between the 67-kDa chimeric toxin and the small GnRH peptides, the similar displacement of the bound GnRH by GnRH-PE$_{66}$ emphasizes the effectiveness of the chimera.

Using different kinds of targeting moieties, a large number of immunotoxins have been generated in the last 20 years by chemical linkage techniques or recombinant DNA technology. The size of the targeting moieties varies widely, ranging from large antibodies to small growth factors, cytokines, and antibody fragments, Nevertheless, to the best of our knowledge, a peptide of 10 amino acids has never before been proposed or used in the construction of chimeric proteins. The ability of a 67-kDa chimeric protein like GnRH-PE$_{66}$ to target cells via a very small portion of the polypeptide and yet retain its original functions, namely binding and internalization, offers new possibilities for designing targeted immunotoxins.

Although the present study clearly demonstrates the presence of GnRH binding sites in certain cancer cells, namely adenocarcinomas, preliminary results utilizing the reverse transcription-polymerase chain reaction and Northern blot techniques (data not shown) suggest that the particular GnRH receptor involved may differ from the previously documented one (29) and we are most likely dealing with an additional GnRH or GnRH-like receptor. Indeed, low affinity-high capacity GnRH binding sites distinct from those of the pituitary GnRH receptor have been demonstrated in ovarian carcinoma (30), renal carcinoma (20), breast carcinoma (2), and placental cells (31). However, this receptor(s) has not been cloned or characterized. It is difficult to reconcile the fact that several cancer cells express the GnRH together with GnRH binding sites (32, 33) and yet GnRH treatment of cancer cells is antiproliferative. We speculate that the functional or physiological significance of the peptide lies in its fine tuning, allowing it to act in an autocrine-inhibitory manner.

In dealing with chimeric toxins, the effect of the nontoxic

moiety is of prime importance, particularly when the full toxic dose is not provided and the targeting portion has a contributive effect on the tumor. Most likely this does not apply to the present case, as GnRH exhibits antitumor activity against a variety of cancer cells (1, 34). If this is the case, then the effect of our chimeric toxin could be an additive one.

However, the killing of tumor cells by GnRH-PE$_{66}$ cannot account for the antiproliferative effect of the GnRH moiety, as its activities are less aggressive and require longer exposure. Indeed, the addition of high concentrations (1.5×10^{-4} M) of GnRH or GnRH analog peptides did not cause any growth inhibition of the cultured tumor cells (data not shown). Weighted against GnRH and analog treatment, the specificity of the chimeric protein, its killing efficiency in marked types of cells, puts the GnRH-PE$_{66}$ in a much more favorable position.

Colon, breast, and lung adenocarcinomas (three of the major malignancies occurring in humans), together with ovarian, endometrial, kidney, and liver adenocarcinomas, account for more then 50% of cancer-related death. The presence of specific GnRH binding sites in all these cancers may suggest a more general role for GnRH and/or GnRH-like peptides in the malignant process. Collectively, our results reveal what we consider to be the Achilles' heel of the malignant growths, a finding that could open up new vistas in the fight against cancer.

In view of the efficient growth inhibition of the above mentioned cancer cells by the GnRH-PE$_{66}$ and its specificity toward non-target cells, the novel chimeric toxin appears to be a promising candidate for cancer treatment.

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